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Cancer by External Imaging

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13. ABSTRACT (Maximum 200 Words) The polypeptide RGD-4C has been shown to localize preferentially on integrins at sites of tumor angiogenesis. The terminal amines of both RGD-4C and RGE-4C peptides were conjugated with NHS-HYNIC and radiolabeled with ^{99m} Tc using tricine. Primarily in the first year, studies in tissue culture were conducted with human umbilical vein endothelial cells (HUVE). Successful radiolabeling of both the RGD-4C and RGE-4C was confirmed by RP and SE HPLC at specific radioactivity of 18 - 20 Ci/ μ mol. Both ^{99m} Tc complexes were stable. At 1 hr, 4 °C and at nM concentrations, the cell accumulation of ^{99m} Tc RGD-4C peptide was as much as 16 times greater than the control. As a check, unlabeled RGD-4C blocked 50% of the binding of ^{99m} Tc labeled RGD-4C. The binding of ^{99m} Tc labeled RGD-4C to purified $\alpha_v\beta_3$ integrin protein was 6.8 fold higher than that of ^{99m} Tc labeled RGE-4C. Thus in the first year of funding, we have shown that a ^{99m} Tc-labeled cyclic RGD-4C peptide shows high selectivity for $\alpha_v\beta_3$ integrin-expressing cells in vitro. In the second year, animal imaging studies were performed with the radiolabeled peptides with poor tumor accumulations. Careful cell binding studies confirmed that one likely reason for the negative results is a low binding affinity of the peptides to the integrin and, especially, a limited number of integrins per cell. Finally, novel alternative labeling methods were investigated to confirm the latter conclusion.				
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Table of Contents

Introduction.....	1
Body.....	2
Key Research Accomplishments.....	7
Reportable Outcomes.....	7
Conclusions.....	11

Introduction

Tumors must establish a neovasculature to grow. To establish a neovasculature, the endothelial cells need to organize themselves into capillaries anchored to the extravascular matrix. The organization of the vascular endothelial cells is accomplished by an interaction of dimerized single-transmembrane glycoproteins, named $\alpha_v\beta_3$ integrin, with triplet peptides Arg-Gly-Asp (RGD) sequenced in the proteins (vitronectin, fibronectin, and von Willebrand factor) of the extracellular matrix. The $\alpha_v\beta_3$ integrins are also expressed on cancer cells and therefore play an important role in the invasion, metastasis, proliferation, and apoptosis of cancer. Consequently, prevention of the tumor angiogenesis could prohibit tumor metastasis. Because the expression of $\alpha_v\beta_3$ integrins are strong on activated endothelial cells but are restricted on normal cells, synthetic RGD containing small peptides have been proposed as antagonists against vascular endothelial cells and tumors growth. The RGD peptides, by binding to $\alpha_v\beta_3$ integrin of either the endothelial or tumor cells, have shown capabilities to inhibit cell-matrix interaction, interrupt signal transmission, affect the cell migration, and cause tumor cell regression or apoptosis.

The detection of early stage cancer is important in preventing cancer cell metastasis. However, the sensitivity and specificity of the current method in detecting breast cancer especially in the early stage are not ideal. Radio labeled RGD peptides could be used as a class of tumor (i.e. tumor angiogenesis) specific markers in cancer detection. Haubner et. al. have reported ^{125}I labeled two RGD peptides, [^{125}I]-3-iodo-D-Tyr4-cyclo(-Arg-Gly-Asp-D-Tyr-Val-) and [^{125}I]-3-iodo-D-Tyr5-cyclo(-Arg-Gly-Asp-D-Phe-Tyr-), as tumor $\alpha_v\beta_3$ integrins antagonists. The accumulation of the ^{125}I -RGD in xenotransplanted melanoma M21 tumor was 1.3~2%ID/g 60 min postinjection and decreased along with time. To improve the tumor uptake of the ^{125}I -RGD, a sugar group was introduced to the cyclic peptides. The tumor uptake of the glycosylated ^{125}I -RGD was maintained at 3%ID/g 60 to 240 min postinjection. However, ^{125}I ($t_{1/2}$ 69.3d, 70keV) is not an adequate radioisotope for nuclear medicine imaging. It has been reported that the aspartic acid residue is highly susceptible to chemical degradation and leads to the loss of biological activity. This degradation could be prevented when the RGD peptide was cyclized via disulfide linkage. In addition to the stability, these cyclic peptides showed higher potency than the linear peptides in inhibiting the attachment of cells to vitronectin. Peptide RGD-4C contains four cysteine residues and forms two disulfide linkages in the molecule. In phage display studies, only phage carrying the RGD-4C peptide accumulated in tumor whereas phage carrying another RGD peptide did not. Our preliminary xenotransplanted tumor imaging test showed that $^{99\text{m}}\text{Tc}$ labeled cyclic RGD-4C, (Cys¹-Cys⁹, Cys³-Cys⁷)H-CDRCRGDCFC-OH, localized preferentially on integrins at sites of tumor angiogenesis (unpublished data). High specific radioactivity labeling would be required for $\alpha_v\beta_3$ integrin imaging if the number of the integrin expressed on tumor vascular cells is limited as reported by others. In this study, we prepared purified $^{99\text{m}}\text{Tc}$ labeled RGD-4C as positive and $^{99\text{m}}\text{Tc}$ labeled RGE-4C, (Cys¹-Cys⁹, Cys³-Cys⁷)H-CDRCGECFC-OH, as control radiotracers at high specific radioactivity, carried out in

vitro $\alpha_v\beta_3$ positive human umbilical vein endothelial (HUVE) cell binding test, and measured the in vivo tumor uptakes of the ^{99m}Tc complex.

Body

Peptides of (Cys¹-Cys⁹, Cys³-Cys⁷)H₂N-Cys-Asp-Cys-Arg-Gly-Asp-Cys-Phe-Cys-COOH (RGD) and (Cys¹-Cys⁹, Cys³-Cys⁷)H₂N-Cys-Asp-Cys-Arg-Gly-Glu-Cys-Phe-Cys-COOH (RGE) were purchased from Advanced ChemTech (Louisville KY, USA). Succinimidyl-6-hydrazinopyridine-3-carboxylic acid (NHS-HYNIC) was synthesized as described. N-tris(Hydroxymethyl)methylglycine (Tricine), N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES), methyl sulfoxide (DMSO) and d₆-DMSO were purchased from Aldrich-Sigma (Milwaukee, WI, USA). Centrifuge (Biofuge *fresco*) was produced by Kendro Laboratory Product (Newtown, CT, USA). The $\alpha_v\beta_3$ integrin protein (MW 237 kDa, stored in a solution of 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2mM MgCl₂, 0.1 mM CaCl₂, 0.2% Triton X-100) was obtained from Chemicon International, Inc. (Temecula, CA, USA). Na $^{99m}\text{TcO}_4$ was obtained from $^{99}\text{Mo}/^{99m}\text{Tc}$ generator (DuPont Pharma, Billerica, MA, USA). C18 Sep-Pak cartridges were produced by Waters Corporation (Milford, MA, USA). Zorbax SB300 C18 reversed phase column (4.6×250 mm, 5 μm) was manufactured by Hewlett-Packard®. C8 reversed phase column (4.6×250 mm, 5 μm) was made by Supelco (Bellefonte, PA, USA). Size exclusion Superdex® Peptide HR 10/30 column (1.0×30 cm) was produced by Pharmacia Biotech AB (Uppsala, Sweden). The high performance liquid chromatography (HPLC) system consisted of a 501 and a 510 pump, a 2487 dual λ channel UV detector (Waters, Milford, MA, USA), and a home-made in-line radiation detector. Radioactive samples were counted in a Packard Cobra II well counter. Proton Nuclear magnetic resonance (^1H and $^1\text{HCOSEY}$ NMR) spectra were recorded on a Varian 400-MHz spectrometer (Palo Alto, CA, USA) with tetramethylsilane as reference. APEX 409M Scintillation camera was manufactured by Elscint (Hackensack, NJ, USA).

Human umbilical vein endothelial (HUVE) cells, endothelial cell basal medium (EBM), and endothelial cell growth medium (EGM) were provided by Clonetics (Walkersville, MD, USA). Human renal adenocarcinoma (ACHN) cell line and human colon cancer cell line (LS174T) were obtained from American Type Culture Collection (Manassas, VA, USA). Minimum Essential Medium (MEM) and fetal bovine serum (FBS) were purchased from Gibco BRL Products (Gaithersburg, MD, USA). N. I. H. Swiss male Nude Mice were provided by Taconic Labs (Germantown, NY, USA). Nembutal was bought from Abbott Laboratories (North Chicago, IL, USA).

Studies Conducted Primarily in the First Year of Funding

The RGD or RGE peptide (1mg, ~1 μmol) dissolved in 1 mL 0.1 M pH 8.0 HEPES buffer was stirred in a 3 mL glass tube at room temperature. To the solution was added 5 μmol (1.4 mg) of NHS-HYNIC dissolved in 50 μL dry DMF (dried over 4 Å molecular sieve). After 1~2

hrs, the coupling solution was loaded on a C18 Sep-Pak cartridge. The cartridge was first washed with 5 mL water and 0.3 mL fractions collected using 20% of MeOH as eluant. Each fraction was analyzed by C18 reversed phase HPLC with water and acetonitrile containing 0.1% TFA as mobile phase with gradient from 100% water to 10% water over 25 min. The retention time (t_R) of native RGD, native RGE, RGD-HYNIC, and RGE-HYNIC were 15.0, 15.2, 15.5 and 15.7 min respectively. The fractions with the highest concentration of RGD or RGE, as indicated by UV absorption at 240 nm and 256 nm, were pooled and lyophilized. Approximately 70% of the theoretical yields were obtained in both cases.

The influence of pH values and concentrations of all reagents on the ^{99m}Tc labeling efficiency to peptides-HYNIC was investigated. The percentage of pertechnetate and reduced ^{99m}Tc oxide colloid were determined by paper chromatography, such as ITLC-SG with saline as mobile phase and Waterman No. 1 paper strip with acetone as developing solvent. The radiochemical purity, recovery, and stability of the ^{99m}Tc labeled peptides-HYNIC were evaluated by C18 HPLC with either water/acetate or water/acetate containing 0.1% TFA as mobile phase. In this way, labeling conditions were optimized.

The general procedure for ^{99m}Tc labeling of the HYNIC conjugated peptides was as follows: to a 3 mL test tube were placed 0.05 μg to 0.2 μg (0.045~0.18 nmol) of RGD-HYNIC or RGE-HYNIC, 15 μL of 100 mg/mL tricine in water, 50 μL 0.25 M pH 5.2 NH_4OAc buffer, 1 to 20 mCi of ^{99m}Tc pertechnetate generator eluant and 4 to 8 μg of SnCl_2 in 10 μL 0.04 N HCl solution. After incubation at room temperature for 20 min, the labeled solution was ready for analysis or purification. For in vivo and in vitro tests, the ^{99m}Tc labeled peptides-HYNIC were always purified by SE HPLC with fractionating in every 0.7 mL. The purity of the fraction with the highest radioactivity was tested before use by re-analysis on SE HPLC with 0.1 M pH 7.2 phosphate buffer (PB). The ^{99m}Tc labeling of native RGD or RGE or NHS-HYNIC was carried out to identify HPLC peaks due to ^{99m}Tc -peptides-HYNIC(tricine) complexes and labeled impurities. Fractions containing the major peaks off C18 and SE HPLC for both ^{99m}Tc labeled peptides were collected separately. The collected fractions were re-injected into both HPLC systems as a check on retention times, radiochemical purity, recovery, and stability.

To establish whether ^{99m}Tc labeled RGD-HYNIC, like the native RGD, showed binding affinity to the $\alpha_v\beta_3$ integrin, both SE HPLC purified ^{99m}Tc -RGD-HYNIC and ^{99m}Tc -RGE-HYNIC were separately mixed with purified $\alpha_v\beta_3$ integrin protein and analyzed by SE HPLC. Before mixing, the $\alpha_v\beta_3$ Integrin protein appeared as a sharp single peak in the UV with retention time of 10.4 min, whereas the purified ^{99m}Tc complexes of either RGD-HYNIC or RGE-HYNIC appeared in the radioactivity profile as a single peak at 22 min. The purified ^{99m}Tc -RGD-HYNIC ($1.7\sim 2.6\times 10^{-14}$ mol) along with the $2\sim 3\times 10^{-12}$ mol of unlabeled RGD-HYNIC, or RGE-HYNIC ($1.9\sim 3.3\times 10^{-14}$ mol) along with $2\sim 3\times 10^{-12}$ mol of unlabeled RGE-HYNIC, were mixed with various molarity of the $\alpha_v\beta_3$ integrin protein (5 μg , 10 μg , and 15 μg respectively, $2.1\sim 6.3\times 10^{-11}$ mol) and injected into the SE HPLC. When mixed, the ^{99m}Tc peak shifted on SE HPLC to that of the $\alpha_v\beta_3$ integrin protein indicating binding

HUVE cells are known to express the $\alpha_v\beta_3$ integrin protein. The cell line was cultured in EGM (500 mL EBM with supplements of 0.5 mL 10 ng/mL human recombinant epidermal growth factor (hEGF), 0.5 mL 1.0 $\mu\text{g/mL}$ of hydrocortisone, and 2.0 mL of a 3mg/mL solution of Bovine Brain Extract (BBE), 0.5 mL of 50 $\mu\text{g/mL}$ Gentamicin, 0.5 mL of 50 ng/mL Amphotericin B) containing 10% FBS in a 37 °C incubator with 5% CO_2 . The cells were harvested by using 0.05% trypsin/0.02% EDTA. After counting by using a trypan blue dye exclusion assay, the cells were suspended in either EGM, or EBM, or PBS at a concentration of 2 to 3×10^6 cells/mL for use.

ACHN and LS174T cell lines were grown in MEM containing 2mM L-glutamine, 1.5mg/L sodium bicarbonate, 0.1 mM non-essential amino acids, 1.0 mM of sodium pyruvate 10% FBS, and 100 mg/mL of penicillin-streptomycin a 37 °C incubator with 5% CO_2 . After harvesting with 0.05% trypsin/0.02% EDTA and rinsing, the cells were suspended in MEM with 0.1% human serum albumin (HAS) to a concentration of $\sim 10^7$ cells/mL for animal implanting.

HUVE cells were cultured in EGM containing 10% FBS. After harvesting, the cells were washed in PBS and suspended in either PBS, or EBM, or EGM at a concentration of 2 to 3×10^6 cells/mL. To the cell suspension was added 0.01 to 15 μCi of the SE HPLC purified $^{99\text{m}}\text{Tc}$ labeled RGD-HYNIC. A parallel binding study was carried out with $^{99\text{m}}\text{Tc}$ -RGE-HYNIC as control. The cell suspension was incubated at 4 °C for 30 min and then centrifuged at 2,000 rpm for 4 min. A 30 μL volume of the supernatant was transferred to a plastic tube for radioactivity counting. The cell pellets were washed with fresh medium three times. Thereafter, the cell pellets were separated from the supernatant and counted. The count^{E5} of the cell pellet was defined as C_{cell} . Count of the supernatant, which was equivalent to the same volume of cell pellet, was defined as C_{medium} . The concentration of $^{99\text{m}}\text{Tc}$ -RGD bound to the cells was plotted against that of free $^{99\text{m}}\text{Tc}$ -Tc-RGD in the medium.

Various concentration (in the range between 50 pM to 58 μM) of native RGD was added to Eppendorf vials containing 2 to 3×10^6 cells suspended in EGM. After incubation at 4°C for 30 min, the $^{99\text{m}}\text{Tc}$ labeled RGD-HYNIC was added and the cells incubated at 4°C for 60 min. The Eppendorf vials were centrifuged and 30 μL of supernatant was transferred for counting. After washing three times with fresh EGM, the cell pellet in the Eppendorf tip was placed in a plastic test tube for counting. The percentage of cell-bound radioactivity relative to the control was plotted against the concentration of the native RGD

About 10 μg of labeled RGD or RGE were purified by SE HPLC. About 100 μL (24 μCi) of the purified $^{99\text{m}}\text{Tc}$ -RGD from the most radioactive fraction (0.7 mL, containing approximate 0.17 μg or 0.16 nmol of RGD-HYNIC) was used for each animal injection. Similarly, the same dosage of $^{99\text{m}}\text{Tc}$ -RGE (containing 0.17 μg or 0.16 nmol RGD-HYNIC) was injected to the two control animals.

LS174T cells (10^6) in MEM containing 0.1% HAS were implanted in one thigh in each of four nude mice. After the tumor grew to approximate 1 cm^2 in any dimension, two mice received $^{99\text{m}}\text{Tc}$ -RGD while another two received a similar dose $^{99\text{m}}\text{Tc}$ -RGE as control. Animals were

imaged at 6 hrs postinjection and were then sacrificed for biodistribution. Radioactivity of the tissue samples was measured against a standard of the injectate.

Studies Conducted Primarily in the Second Year of Funding

As described above, we have previously prepared a cyclic RGD peptide with HYNIC conjugation and labeled the conjugate with ^{99m}Tc to high specific radioactivity using tricine as coligand. The radiolabeled RGD-4C displayed high binding specificity to either purified $\alpha_v\beta_3$ integrin protein or to human umbilical vein endothelial (HUVE) cells. However, the tumor uptake of the label in xenotransplanted human colon and human renal tumors (transplanting LS174T and ACHN cell lines respectively) in mice was modest possibly because the number of the integrin proteins expressed in the tumor region was small combined with high serum protein binding resulting from the presence of tricine in the radiolabeled conjugate. The objective of this latest investigation was to determine by in vitro cell studies whether the serum protein binding of the RGD label was an important factor in the previous negative results. Therefore, the EDDA and tricine complexes of ^{99m}Tc -RGD-HYNIC peptide were prepared, purified, and their stability, lipophilicity, and HUVE cells binding properties were compared.

The ^{99m}Tc labeling of the RGD/E peptides with tricine as co-ligand was accomplished as before using 0.05 μg of HYNIC-RGD/E conjugate in 5 μL water, 15 μL of 100 mg/mL tricine in water, 50 μL 0.25 M NH_4OAc buffer pH 5.2, 1 to 5 mCi of ^{99m}Tc pertechnetate generator eluant and 4 to 8 μg of SnCl_2 in 10 μL 0.04 N HCl solution were mixed. After incubation at room temperature for 20 min, the radiolabels were analyzed and purified by HPLC.

The ^{99m}Tc labeling of the RGD/E with EDDA as co-ligand proceeded as follows: 0.5 μg HYNIC-RGD/E conjugate in 5 μL of water, 50 μg EDDA in 50 μL water, 1 to 5 mCi ^{99m}Tc pertechnetate generator in 10~50 μL saline, and 2~3 μg SnCl_2 in 5~7 μL water were mixed. After incubation at room temperature for 30 min, the ^{99m}Tc labels were analyzed and purified by SE HPLC by fractionation as stated above. The fraction with the highest radioactivity was used for in vitro experiments.

The preparation of HUVE cells was described previously. Briefly, the HUVE cell line was cultured in EGM [500 mL EBM with supplements of 0.5 mL of 10 ng/mL human recombinant epidermal growth factor (hEGF), 0.5 mL of 1.0 $\mu\text{g}/\text{mL}$ of hydrocortisone, and 2.0 mL of a 3 mg/mL solution of Bovine Brain Extract (BBE), 0.5 mL of 50 $\mu\text{g}/\text{mL}$ Gentamicin, 0.5 mL of 50 ng/mL Amphotericin B] containing 10% FBS in a 37 °C incubator with 5% CO_2 . The cells were harvested in 0.05% trypsin/0.02% EDTA. After cell counting by using a trypan blue dye exclusion assay, the cells were suspended in EGM at a concentration of $2\sim3\times10^6$ cells/mL for use. It is assumed that the packed volume of 500 million HUVE cells is equal to 1 mL.

Purified radiolabels at various concentrations (70 μL , 0.03-33 μCi , equivalent to $64\times10^{-15}\sim64\times10^{-18}$ mol) were added to the cell suspension (0.7-1 mL, $2\sim3\times10^6$ cells/mL). The

cell suspension was incubated at 4 °C for a certain period and then centrifuged at 2,000 rpm for 4 min. A 30 µL volume of the supernatant was transferred to a plastic tube for radioactivity counting. The cell pellets were washed in ice-cold fresh medium 3 times and then separated from the supernatant for counting. The count of the cell pellets was defined as C_{cell} , while those of an equal volume of the supernatant were defined as C_{medium} .

A 100 µL volume of each purified radiolabel (5-10 µCi, 9.6-19 fmol) was mixed with an equal volume of 5% BSA PBS solution and incubated at 37 °C for 1 hr before analysis by SE HPLC to measure binding.

In triplicate, a 100 µL aliquots of each purified label in Eppendorf vials was diluted with 400 µL of PBS, mixed with 500 µL of n-Octanol, and vortexed at room temperature for 1 min. After the phases were given time to separate, the vials were centrifuged at 12,000 rpm for 4 min and 3×10 µL of the aqueous phase and 3×100 µL of the organic phase from each Eppendorf vial was transferred to plastic tubes for radioactivity counting. The partition ratios of the labels were calculated by dividing the counts of the organic phase with that of aqueous phase per unit volume.

In quintuplet, 2.1×10^6 HUVE cells were suspended in 0.7 mL EGM medium and were incubated with 100 µL of each purified radiolabel (11~13 µCi, equivalent to 21~25 fmol) at 4 °C for 1 hr. After centrifugation at 2,000 rpm for 4 min, duplicate 30 µL samples of the supernatant were transferred for radioactivity counting. The cell-pellets were washed 3 times with 0.5 mL ice-cold PBS. The protocol for protein isolation was that of MRC Inc (Molecular Research Center, Inc., Cincinnati, OH. <http://www.mrcgene.com/tri.htm>). Briefly, the cells were homogenized by adding 0.4 mL of TRIReagent. After incubating at room temperature for 5 min, the homogenate was vortexed for 15 sec following the addition of 0.05 ml 1-bromo-3-chloropropane. The mixture was incubated at room temperature for 15 min and then centrifuged at 12,000 g for 15 min at 4 °C. The lower red phenol-chloroform phase and interphase were isolated from the aqueous phase. The DNA was precipitated from the interphase and organic phase by adding 0.12 mL ethanol and the proteins were then precipitated by adding 0.6 ml of isopropanol to the phenol-ethanol supernatant. After incubation at room temperature for 15 min, the samples were centrifuged at 12,000 rpm for 10 min at 4 °C. The supernatant was removed and the protein pellet was washed with a solution of 0.3 M guanidine hydrochloride in 95% ethanol. The samples in the washing solution were incubated at room temperature for 20 min and then centrifuged at 7,500 g for 5 min at room temperature. The protein-pellets were then washed by 1 ml of ethanol. Following 20 min incubation at room temperature, the washing solution was centrifuged at 7,500 g for 5 min at 25 °C. The radioactivity of the cell pellets was then counted.

Key Research Accomplishments (Both Years)

- A method of radiolabeling a cyclic RGD peptide with ^{99m}Tc was developed and characterized. The radiolabel was shown to be stable in various environments. Most importantly, by the use of a similar RGE control peptide, the radiolabeled RGD was shown to bind specifically to $\alpha_v\beta_3$ expressing cells.
- As a results of the low numbers of $\alpha_v\beta_3$ receptors thought to be present in tumors, the method of radiolabeling the RGD was modified to provide specific radioactivities sufficiently high for a test of in vivo tumor imaging.
- Cell accumulations studies at different dosages of labeled RGD peptide suggested that the number of integrins per tumor cell to be limited.
- Possibly for this reason, tumor imaging studies in nude mice provided disappointing results.
- The method of radiolabeling was investigated by comparing tricine and EDDA as coligands for the HYNIC-coupled peptides to exclude radiolabeling as a factor in the binding studies.
- Thus far, all labeling methods have pointed to the same conclusion that $\alpha_v\beta_3$ integrin concentration may be limited.

Reportable Outcomes (First Year)

C18 Sep-Pak cartridges were used successfully as a simple and effective method to separate the labeled peptides from most of the impurities, such as NHS-HYNIC, hydrated HYNIC, HEPES, and solvents such as DMF. After the purification, the ratio of peptide-HYNIC to uncoupled peptides was in the range of 6 to 10 as indicated by UV absorbance on C18 HPLC. The presence of unlabeled peptides did not significantly affect the ^{99m}Tc labeling of HYNIC coupled peptides.

The pH value appeared to be critical for successful labeling. For example, the labeling efficiency for both peptides was higher in acidic medium than in neutral or basic medium. The C18 HPLC radiochromatograms with water/acetonitrile as mobile phase of ^{99m}Tc labeled RGD show retention time 15.7 min and 15.9 min for RGE. The ^{99m}Tc labeling of RGD or RGE produced broad peaks (retention times from 12 to 17 min) on C18 HPLC with 0.1% TFA in the mobile phase. Free NHS-HYNIC did not show significant ^{99m}Tc labeling under the same conditions. Minor peaks at 12.6 to 13.3 min are due to impurities and were not stable in acidic medium, such as pH 5.2 acetate buffer or water/acetonitrile containing 0.1% TFA. When the labeling was carried out in neutral media, such as water or 0.1 M pH 7.2 PB, the extent of impurities could be as high as 44% as detected. Lower levels of impurities (11 to 22%) formed when the labeling was carried out in 0.25 M pH 5.2 acetate buffer. Paper chromatogram indicated that there was little free ^{99m}Tc remaining but approximate 10~15% of the ^{99m}Tc was present as colloids. Approximately 30~50% of the ^{99m}Tc labeled peptides-HYNIC was accumulated on the C18 or SE column during the HPLC purification. It is assumption that the ^{99m}Tc colloid, which

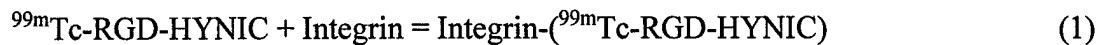
was not washed off but accumulated on either C18 or SE column, would adhere part of the ^{99m}Tc labeled peptides on the HPLC column as well. The formation of radiocolloid could be diminished through the use of ^{99m}Tc -gluconate or ^{99m}Tc -tartrate as transfer ligands, but the labeling efficiency became worse possibly due to HYNIC-Tricine were not significant stronger chelating agents than gluconate or tartrate.

It was primarily to prevent introducing ^{99m}Tc colloids that the ^{99m}Tc labeled RGD and RGE were SE HPLC purified before use. The radiochemical purity of the purified ^{99m}Tc complexes was confirmed by re-injection. Moreover, the purified ^{99m}Tc complexes was also analyzed by C18 HPLC with 0.1% TFA in the mobile phase. The radiochromatogram exhibited a major sharp peak with a retention time of 15.4 min and therefore identical to that of the unpurified sample. This indicated that the major peak on both C18 and SE HPLC was due to the desired ^{99m}Tc complex.

The unpurified ^{99m}Tc -peptides were stable in the labeling solution for over 20 hrs in that the radiochemical purity, recovery, and the percentage of ^{99m}Tc colloid remained unchanged on standing. However, the radiochemical purity and recovery of the ^{99m}Tc complexes were greatly improved by re-injecting the C18 HPLC purified (with 0.1% TFA in the mobile phase) ^{99m}Tc complexes into the same HPLC and SE HPLC system. The radiochemical purity and recovery of both ^{99m}Tc labeled RGD-HYNIC and RGE-HYNIC were over 95% when they were re-injected into C18 HPLC with or without 0.1% TFA in the mobile phase. Single peaks were detected when they were injected into SE HPLC with a retention time of 22 min and recovery of 78% and 85% respectively. This had further indicated that the ^{99m}Tc complexes of peptides-HYNIC were stable in either acidic and neutral media (water or 0.1 M pH 7.2 PB).

High specific radioactivity could be achieved by labeling as small a dosage as possible of peptides with a large excess of ^{99m}Tc . Technetium-99m labeling of both peptides-HYNIC with concentrations as low as 0.5 to 4.5 nmol was investigated. By C18 HPLC, the minimum molarity of the peptide providing adequate radiolabeling was about 4.5 pmol (5 ng), since the yield of ^{99m}Tc -RGD-HYNIC was only 10% (50 μCi) under these conditions. In practice, 45 to 90 pmol (50 to 100 ng) of the peptide-HYNIC were used for ^{99m}Tc labeling to provide sufficient radiotracer for both cell binding and animal studies. Labeling of higher concentrations of peptide-HYNIC generally resulted in higher radiochemical yield (Table 1), but the radiochemical recovery from C18 HPLC remained unchanged (52~55%) because of the presence of ^{99m}Tc colloid. To optimize specific radioactivity, especially for cell binding studies, ^{99m}Tc labeling of 45 pmol (50 ng) RGD or RGE with increasing amount of ^{99m}Tc was investigated. The maximum specific radioactivity was approximately 20 to 30 Ci/ μmol for both RGD and RGE, depending somewhat on the quality of ^{99m}Tc pertechnetate generator elute. To ensure sufficient quantities of purified labeled peptides (200~400 μCi in 0.7 mL phosphate buffer) for 5 animal injection, 90 to 135 pmol (0.1~0.15 μg) peptide-HYNIC was used for each labeling.

By comparison with ^{99m}Tc -RGE, the ^{99m}Tc complex of RGD showed specific binding to $\alpha_v\beta_3$ integrin protein. The binding percentage of the ^{99m}Tc -RGD-HYNIC to $\alpha_v\beta_3$ integrin protein increased from 7.5% to 20.4% along with the increase of the concentration of the integrin (0.2~0.5 μmol), whereas no significant shift had been seen for ^{99m}Tc -RGE-HYNIC. It was noted that the majority of the ^{99m}Tc -RGD-HYNIC remained unbound even though the molarity of the integrin used was greater than that of either the ^{99m}Tc complex or the unlabeled RGD-HYNIC. This might be due to the weak association constant of the ^{99m}Tc -RGD-HYNIC with the integrin. It is assumed that the binding of the ^{99m}Tc -RGD-HYNIC to the $\alpha_v\beta_3$ integrin protein follows a thermodynamic equilibrium shown in equation 1:



Let K is the equilibrium constant, and equation 1 can be rewritten as equation 2:

$$\text{Log}\{[^{99m}\text{Tc-RGD-HYNIC}]_b/[^{99m}\text{Tc-RGD-HYNIC}]_f\} = \text{LogK} + \text{Log}[\text{Integrin}]_f \quad (2)$$

Where $[^{99m}\text{Tc-RGD-HYNIC}]_b$ and $[^{99m}\text{Tc-RGD-HYNIC}]_f$ are the concentrations of the bound and the free ^{99m}Tc -RGD-HYNIC, while $[\text{Integrin}]_f$ was the concentration of the free $\alpha_v\beta_3$ integrin protein. The log~log plot of the concentration ratio of bound to free ^{99m}Tc -RGD-HYNIC vs. the concentration of the free integrin exhibited a straight line with an intercept of 6.83 on the y-axis, which corresponded to the value of LogK. Accordingly, the association constant of ^{99m}Tc -RGD-HYNIC with $\alpha_v\beta_3$ integrin protein is approximately $7 \times 10^7 \text{ M}^{-1}$.

SE HPLC purified ^{99m}Tc labeled RGD and RGE were used as positive and control radiotracers for HUVE cells binding respectively. The results revealed that the ^{99m}Tc complexes exhibited important differences in cells binding in the expected direction. For example, ^{99m}Tc -RGD showed 5.3 to 16 (n=4) fold higher binding than that of the control. Cell uptake of both ^{99m}Tc complexes improved when the specific radioactivity increased (Table 3). However, this improvement in $C_{\text{cell}}/C_{\text{medium}}$ was surprisingly small in comparison to the large change in specific radioactivity. Furthermore, the type of media influenced the binding of ^{99m}Tc -RGE but not ^{99m}Tc -RGD. The $C_{\text{cell}}/C_{\text{medium}}$ ratios of ^{99m}Tc -RGD in HUVE cells in the three media were similar, while those of ^{99m}Tc -RGE to the cells varied with media. The binding of labeled RGD vs. labeled RGE was highest in EGM (6.9 fold) and lowest in PBS with intermediate results in EBM.

It was important in this research to estimate the number of $\alpha_v\beta_3$ integrin expressed on average per cell since this value is a direct determinant of the amount of ^{99m}Tc labeled RGD which will bind. However, the cell binding data did not produce a straight line when subjected to

a Scatchard Plot^{R10} therefore neither the number of $\alpha_v\beta_3$ integrin expressed on the cell nor the binding constant of the ^{99m}Tc -RGD with the integrin could be estimated by this method.

Reportable Outcomes (Second Year)

With tricine as co-ligand, usually 50 ng of the RGD/E was required for binding 1 to 5 mCi ^{99m}Tc and specific radioactivities as high as 20 Ci/ μmol were achieved(34). With this co-ligand, approximately 3~5% unreduced ^{99m}Tc appeared at 3.5 min on HPLC for both RGD and RGE but with EDDA, much more unreduced ^{99m}Tc was detected for both RGD and RGE. The extent of contamination with unreduced ^{99m}Tc was not improved by adding more stannous ion to the labeling solution. By paper chromatography, approximately 10~15% (tricine) and 7~10% (EDDA) of the ^{99m}Tc was presented as colloids for both RGD/E. The purity and stability of the HPLC purified radiolabels were evaluated by re-injection into reverse phase and size exclusion HPLC respectively. The labels were stable in the labeling solution, in water/acetonitrile mixture containing 0.1% TFA, and in 0.05 M phosphate buffer for over 12 hrs without significant change.

The cell accumulation of the radiolabels was evaluated by comparing the radioactivity in cells with that in a supernatant of the same volume. The RGD labels (RGD/tricine and RGD/EDDA) always exhibited higher accumulation in HUVE cells over the RGE labels. The cell accumulation of RGD over RGE averaged 6-fold higher with tricine and 28 fold higher with EDDA. The accumulation of the labeled RGD in cells increased with the concentration of the RGD/tricine or RGD/EDDA in the medium. The amount of RGD/tricine or RGD/EDDA that accumulated in the cells increased slowly when the concentration of the peptides was less than 4 pM, but then increased sharply at higher concentrations. Similar to the RGD/tricine, straight lines are seen when the log concentrations of the RGD/EDDA or the RGE/EDDA in the cells is plotted against the log concentration in the medium. The straight line shows that a good correlation exists between the concentration of the label accumulated in the cell and that remaining in the medium over the entire concentration range. This implies that most of the RGD/EDDA accumulated in HUVE cells under the experimental condition of this investigation is probably not specifically bound to the $\alpha_v\beta_3$ integrins just as was observed earlier by us in the case of tricine as co-ligand. Most of the labeled RGD may have accumulated nonspecifically in the cytosol after the integrins were saturated by the non-labeled and labeled RGD. If so, then the saturation of the integrins occurred even at the lowest incubation concentration of RGD ($\sim 7 \times 10^{-12}$ M).

The lipophilicity of the radiolabels was evaluated by their partition ratios between n-Octanol and PBS. All four ^{99m}Tc complexes showed low ratios (0.0010 \pm 0.0001 for RGD/tricine, 0.0029 \pm <0.0001 for RGE/tricine, 0.0019 \pm 0.0001 for RGD/EDDA, and 0.0019 \pm 0.0001 for RGE/EDDA, n = 3). Thus the partition ratios with tricine were almost 3 fold higher for RGE compared to RGD while that of RGD/E were similar with EDDA. Since a low partition ratio implies low lipophilicity, these results suggest that the lipophilicity of RGD was lower than that of RGE when labeled with tricine as coligand but similar when labeled with EDDA.

The binding of the radiolabels to BSA was investigated by SE HPLC. The tricine complexes showed 80% and 90% binding for RGD/E respectively while for the EDDA complexes showed

only 10% binding . The tricine complexes therefore showed 8~9 fold higher binding to BSA than that of the EDDA complexes.

By comparing the total radioactivity added in the cell incubation with that associated with isolated protein, it was possible to show that the percent radiolabel bound to total cellular proteins for 2.1×10^6 HUVE cells were $0.059\% \pm 0.003\%$ (RGD/tricine), $0.014\% \pm 0.006\%$ (RGE/tricine), $0.036\% \pm 0.009\%$ (RGD/EDDA), and $0.012\% \pm 0.006\%$ (RGE/EDDA). That the protein-binding percentages for the two RGE complexes were similarly low suggests that the binding of both these two peptides were non-specific. In contrast, the protein binding percentages of both RGD labels were higher, indicating $\alpha_v\beta_3$ integrin protein specific binding. Considering the competition of the unlabeled RGD in the purified RGD labels, it is possible that the binding affinity of the RGD/tricine and RGD/EDDA may be identical. Since the specific radioactivity of the RGD/EDDA was estimated to be 1/10th that of RGD/tricine, the concentration of unlabeled RGD may be expected to be 10 fold higher in the purified RGD/EDDA over that in the RGD/tricine peptide preparations. Thus the unlabeled RGD would compete with the binding of the RGD labels to the integrin proteins and this competition would be more severe for RGD/EDDA than for RGD/tricine. Considering that the binding of the RGE complexes was non-integrin-specific and if this portion is subtracted from the binding of the RGD labels, it can be calculated that roughly one molecule of RGD/EDDA and three molecules of RGD radiolabel were specifically bound to the integrin proteins of a single cell.

Conclusion(First Year)

Current methods of detecting cancer have both low specificity and sensitivity. For example, in the case of breast cancer, although screening mammography results in early detection and reduces death from this disease, it has a low positive predictive value and a 60~90% false positive rate that leads to pain, morbidity and potential disfigurement associated with an estimated 500,000 unnecessary breast biopsies. There is, therefore, a pressing need for a widely available, non-invasive and the number of cancer-negative biopsies. Although scintimammography using technetium-99m sestamibi improves the clinical management of patients with suspected breast cancer, It is likely that further gains in specificity and sensitivity can be achieved by using receptor-based imaging agents that target specific features of breast cancer tissue such as its neovasculature. In breast cancer as with all other neoplastic diseases, tumor growth and metastasis require persistent new blood vessels without which tumor cells become necrotic or apoptotic. Among the molecular markers associated with neovascular angiogenesis is the $\alpha_v\beta_3$ integrin, a protein which is absent or barely detectable in established blood vessels but is concentrated in forming blood vessels. Thus, the early and highly selective appearance of this integrin in tumor makes it a useful target for tumor imaging. The cyclic cysteine-rich polypeptide RGD-4C molecules were designed as $\alpha_v\beta_3$ integrin specific marker for tumor angiogenesis imaging with RGE-4C as control. Our preliminary result has shown that the ^{99m}Tc labeled RGD peptide localized preferentially on integrins at sites of tumor

angiogenesis and thus can be an excellent choice for the delivery of radioactivity such as ^{99m}Tc for the early detection of the disease. However, as it has been reported in the literature, the number of $\alpha_v\beta_3$ integrin proteins expressed on endothelia or tumor cells may be small because the uptake of the radiotracers (id%/g at 1 to 24 hrs) in all the organs especially in the xenograft tumor were small. If so a high specific radioactivity will be required for imaging to diminish the saturation of integrin binding with unlabeled RGD-HYNIC and RGD.

Technetium-99m is a metal ion and is usually complexed by ligands attached to biological molecules. Thermodynamically, excessive chelator has to be applied for the chelation of technetium. Accordingly, it is difficult to achieve high specific radioactivity with ^{99m}Tc labeling although there are successful examples of high specific labeling with this radionuclide. A unique method of producing high specific ^{99m}Tc labeled peptide has been reported by Baidoo et. al. After the radiolabeling of a peptide containing a diaminedithiol chelator, the unlabeled peptide was removed on a iodoacetamide-derivatized gel resulting in a high specific radioactivity of at least 100 Ci/ μmol (the remained unlabeled peptide was below the detection limit). Another successful approach to high specific ^{99m}Tc labeling of small peptides containing HYNIC as chelator was reported by Liu et. al.. The radiochemical yield was $\geq 90\%$ and the specific radioactivity was ≥ 20 Ci/ μmol . HYNIC-tricine can complex ^{99m}Tc at room temperature and was used for antibody labeling in this laboratory.

The coupling of NHS-HYNIC to RGD and RGE was convenient and the purification of RGD-HYNIC and RGE-HYNIC by C18 Sep-Pak cartridge was simple and reproducible. Technetium-99m labeling of 45~135 pmol of the RGD-HYNIC and RGE-HYNIC, only 1% to 0.1% the amount of peptides usually used for ^{99m}Tc labeling, was successfully performed in this laboratory. The free RGD-HYNIC and RGE-HYNIC in the labeling solution was undetectable by UV because their concentrations were too low. High specific radioactivity (approximate 20-30 Ci/ μmol) was achieved. Thus, this specific radioactivity is similar to that reported by others^{D13,14}. This high specific activity was achieved despite the presence of unlabeled RGD-HYNIC or RGE-HYNIC in each preparation. The specific radioactivity was depended on the quality of the generator eluant used. One further problem in the ^{99m}Tc labeling of low concentration of peptide-HYNIC was the unavoidable formation of ^{99m}Tc colloids. In spite of this, a radiochemically pure ^{99m}Tc complex was produced as shown by a single major peak on C18 and SE HPLC with radiochemical recovery over 52%. Combining with SE HPLC purification, the purity of the ^{99m}Tc labeled peptides for cell binding and in vivo animal tests was documented in this study.

Despite the difficulties in structurally characterization of the ^{99m}Tc -peptides-HYNIC by NMR and MS because weighable amount of the ^{99m}Tc complexes was technically not obtainable, there are sufficient reasons to believe that the major peak shown on either C18 or SE HPLC was the desired complex. First, C18 HPLC radiochromatograms revealed that the retention times of ^{99m}Tc -RGD-HYNIC (retention time 15.7 min) and ^{99m}Tc -RGE-HYNIC (15.9 min) were greater than that of the unlabeled RGD-HYNIC (15.5 min) and RGE-HYNIC (15.8 min) because the lipophilicity of the ^{99m}Tc complexes were higher than the unlabeled peptides-HYNIC. Second,

the yields of ^{99m}Tc labeling of native RGD and RGE were low (less than 10% of the total radioactivity used), and it was even much lower in the presence of free RGD-HYNIC or RGE-HYNIC. Third, the non-specific ^{99m}Tc labeled RGD and RGE showed broad peaks on C18 HPLC and were unstable in acidic solvents, such as water/acetonitrile containing 0.1% TFA, whereas the ^{99m}Tc complexes of the peptides-HYNIC were stable either in acidic or neutral solvents, such as water and 0.1 M phosphate buffer.

The ^{99m}Tc labeled RGD and RGE were purified by SE HPLC with 0.1 M pH 7.2 PB as eluant. The purity and stability of the purified ^{99m}Tc complexes were tested by re-injection to SE and C18 HPLC. The results confirmed first that the major peaks which showed on C18 and SE HPLC were the same complexes, second that the purification was successful and reproducible and third that the complexes were stable in either acidic and phosphate buffer which would guarantee that they had no change in cell binding and in vivo animal tests.

The binding percentage of the ^{99m}Tc -RGE-HYNIC to HUVE cells was always smaller than that of the ^{99m}Tc -RGD-HYNIC almost certainly because the former does not specifically bind to the $\alpha_v\beta_3$ integrin. However, the binding percentage of the control ^{99m}Tc complex was medium dependent while the ^{99m}Tc -RGD-HYNIC was not. The binding differentials of ^{99m}Tc labeled RGD-HYNIC and RGE-HYNIC was highest (6 to 16 folds, $n=4$) when the cells were suspended in complete medium (EGM). A slight cell binding differential (3 fold) of the two ^{99m}Tc complexes had been seen when the binding study was carried out in basal medium (EBM), whereas there was only a 1.5 fold differential when the study was done in PBS. At the last situation, the binding of the ^{99m}Tc -RGD to HUVE cells could be non-specific as well because the number of the binding sites was small and easily to be saturated by ^{99m}Tc labeled and unlabeled RGD-HYNIC. The small number of $\alpha_v\beta_3$ integrins expressed on vascular or tumor cells would result in poor radioactivity uptake and poor target/non-target identification. This may explain the low organ and tumor uptake of radioactivity measured in the four mice bearing LS174T tumor. The low organ-take-up results has also been reported by other researchers with ^{125}I labeled RGD peptides as $\alpha_v\beta_3$ markers.

The ^{99m}Tc complexes of RGD-HYNIC and RGE-HYNIC showed the expected differences in $\alpha_v\beta_3$ integrin protein binding between the study and control peptides. As indicated by SE HPLC, the binding percentage of the ^{99m}Tc -RGD-HYNIC rose from 7.5% to 20.4% when the concentration of the integrin increased from 0.2 to 0.5 μM . No significant shift had been detected in the case of ^{99m}Tc -RGE-HYNIC. The small percentage shift of ^{99m}Tc -RGD-HYNIC by $\alpha_v\beta_3$ integrin might be mainly due to the weak association constant ($7 \times 10^7 \text{ M}^{-1}$). The manufacturer guarantees that the contamination of the integrin by matrix proteins containing RGE tripeptides during manufacturing was extremely little. Nevertheless, the binding difference of ^{99m}Tc complexes on $\alpha_v\beta_3$ integrin protein was inconsistent with that seen in the HUVE cell binding tests. The data on Table 3 show a slight increase in cell binding of the ^{99m}Tc -RGD-HYNIC when the specific radioactivity of the complex increased greatly. Usually, the binding of antigen-antibody or receptor-ligand exhibits an increase in binding with dosage until saturating concentrations are reached. However, when the data of the table are plotted, the curve is quite

different, showing no increase in binding with dosage until a point is reached in which binding greatly increases with concentration. This suggests that the binding of the ^{99m}Tc -RGD-HYNIC to the HUVE cells under our conditions was not specific. This non-specific binding might occur even early when 3×10^6 cells were incubated with $0.015 \mu\text{Ci } ^{99m}\text{Tc}$ -RGD-HYNIC ($3 \times 10^{-14} \text{ M}$, 1.7×10^7 molecules) along with $4.8 \times 10^{-12} \text{ M}$ (3×10^9 molecules) of unlabeled RGD-HYNIC probably because the $\alpha_v\beta_3$ integrin was saturated by those molecules. Assuming that 1) the binding sites of the 3×10^6 cells were saturated by the unlabeled RGD-HYNIC ($4.8 \times 10^{-12} \text{ M}$, 3×10^9 molecules) at this point, 2) the concentration of the RGD-HYNIC bound to the cell was approximately equal to that in the supernatant, then the concentration of the unlabeled RGD-HYNIC bound to the cells would be $4.8 \times 10^{-12} \text{ M}$ in maximum. This may suggest that the concentration of the $\alpha_v\beta_3$ integrin expressed on the HUVE cells could not be greater than Pico molar. Taken together, the ^{99m}Tc labeled RGD peptides may have limitation as markers for tumor imaging because 1) the number of the $\alpha_v\beta_3$ integrin expressed on the HUVE cell was little, 2) the binding constant of ^{99m}Tc -RGD-HYNIC to the integrin was weak ($7 \times 10^6 \text{ M}^{-1}$). These factors would cause limited specific binding of the ^{99m}Tc complex to the integrin. For instance, the concentration of ^{99m}Tc -RGD-HYNIC specifically bound to the $\alpha_v\beta_3$ integrin of 10^6 tumor cells (approximate $2 \mu\text{L}$ in volume) implanted in a 25 g nude mice (has approximately 1.4 mL blood) would be $2 \times 10^{-16} \text{ M}$ only, when $20 \mu\text{Ci}$ purified ^{99m}Tc -RGD-HYNIC ($\sim 4 \times 10^{-14} \text{ mol}$, $2.8 \times 10^{-11} \text{ M}$) is assumed to be injected. Simply put, only 240 molecules of ^{99m}Tc -RGD-HYNIC would bind to the $\alpha_v\beta_3$ integrin of the 10^6 tumor cells at this situation. The radioactivity exceeds this level could be caused by non-specific binding. The $\alpha_v\beta_3$ integrins expression on HUVE and ACHN cell types have been confirmed but not quantified.

Conclusion(Second Year)

The cell binding characteristics of the ^{99m}Tc labeled RGD/E with tricine and EDDA as co-ligands were investigated this year. The objective of this investigation was to determine by in vitro cell studies whether the protein binding of the RGD label with tricine was an important factor in the previous negative results. However, in spite of the difference in BSA binding percentage, the RGD/tricine and RGD/EDDA were almost identical in $\alpha_v\beta_3$ integrin proteins binding affinity. Regardless of the difference in specific radioactivity, the RGD/tricine and RGD/EDDA had shown similarity in the levels of cell accumulation indicating that these co-ligands and the alternative labeling method did not significantly change the cell accumulation mechanism of the ^{99m}Tc -RGD complexes. Combining with the previous data from the first year with the data from the second year, it may conclude that most of the RGD labels associated with the cells might be presented in the cytosol but not bound by the $\alpha_v\beta_3$ integrins. Binding of RGD to $\alpha_v\beta_3$ integrins may cause the internalization of the RGD labels to the cells. Finally, in agreement with our earlier study of the first year, quantitation of the cell binding results again suggests that the number of $\alpha_v\beta_3$ integrin proteins per cell may be limited. The density and accessibility of the $\alpha_v\beta_3$ integrins expressed on the blood vessel cells and the tumors will be crucial for the development of radiolabeled RGD based

imaging agents. As a result of these observations, it was assumed that RGD/EDDA may not behave significantly different from RGD/tricine in the xenografted tumor accumulation and for this reason tumor animal studies were considered unreasonable.

Publications

Su Z., Zhu Z., Liu G., Gupta S., Liu C., Zhang Y., Liu N., Rusckowski M., Hnatowich DJ. In vitro and in vivo evaluation of a technetium-99m labeled RGD peptide as a specific marker of $\alpha_v\beta_3$ integrins for tumor imaging Bioconj Chem (in press).